Carbonyl Sulfide and Carbon Dioxide as New Substrates, and Carbon Disulfide as a New Inhibitor, of Nitrogenase†

Lance C. Seefeldt,*,‡ Madeline E. Rasche,§ and Scott A. Ensign‡

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322-0300, and Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received November 23, 1994; Revised Manuscript Received February 8, 1995⊗

ABSTRACT: Nitrogenase is the metalloenzyme responsible for the biological reduction of N_2 to NH_3. Nitrogenase has been shown to reduce a variety of substrates in addition to N_2 and protons. General properties of alternative substrates for nitrogenase are the presence of N−N, N−O, N−C, and C−C triple or double bonds. In the present work, we demonstrate that *Azotobacter vinelandii* nitrogenase can reduce both C−S and C−O bonds. Nitrogenase was found to reduce carbonyl sulfide (COS), to CO and H_2S at a maximum rate of 37.2 ± 2.0 nmol min^{-1} (mg of protein)$^{-1}$ with a K_m of 3.1 ± 0.6 mM. The formation of CO from nitrogenase reduction of COS was monitored spectrophotometrically in real time by following the formation of carboxyhemoglobin. In this assay, the change in the visible absorption spectrum of reduced hemoglobin upon binding CO provided a sensitive way to quantify CO formation and to remove CO, which is a potent inhibitor of nitrogenase, from solution. COS reduction by nitrogenase required the molybdenum−iron protein (MoFeP), the iron protein (FeP), and MgATP. The reduction reaction was inhibited by MgADP, acetylene, and N_2, while H_2 was not an inhibitor of COS reduction. The pH optimum for COS reduction was 6.5. Nitrogenase was also found to reduce carbon dioxide (CO_2) to CO and H_2O. CO_2 was reduced at a maximum rate of 0.8 ± 0.07 nmole min^{-1} (mg of protein)$^{-1}$ with a calculated K_m for CO_2 of 23.3 ± 3.7 mM. COS and CO_2 did not inhibit proton reduction by nitrogenase or total electron flow through nitrogenase. In contrast, the COS and CO_2 analog carbon disulfide (CS_2) was found to be an inhibitor of nitrogenase reduction reactions and total electron flow. CS_2 was a rapid-equilibrium, mixed-type inhibitor with respect to acetylene reduction (K_{i1} = 2.9 ± 0.6 mM and K_{i2} = 12.1 ± 2 mM) and also inhibited both proton and COS reduction.

Nitrogenase catalyzes the reduction of N_2 to NH_3 according to the reaction

$$N_2 + 8H^+ + 16MgATP + 8e^- \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i \quad (1)$$

Nitrogenase is a complex metalloenzyme consisting of two separable component proteins, the molybdenum−iron protein (MoFeP)[1] and the iron protein (FeP). The MoFeP contains the site of substrate binding and reduction, which is thought to be at a molybdenum−iron cofactor (FeMoco) [see for reviews Burris (1991), Smith and Eady (1992), Dean et al. (1993), Mortenson et al. (1993), Rees et al. (1993), Kim and Rees (1994)]. The MoFeP also contains [8Fe-(7−8)S] clusters (8Fe or P cluster) which appear to mediate electron transfer from the FeP to the FeMoco (Kim & Rees, 1992a,b; Bolin et al., 1993; Lowe et al., 1993). The FeP is a homodimeric protein containing a single [4Fe-4S] cluster and two nucleotide binding sites, one on each subunit (Georgiadis et al., 1992; Mortenson et al., 1993). A consensus model for nitrogenase-catalyzed reduction reactions holds that the FeP transfers a single electron from its [4Fe-4S] cluster to the MoFeP (probably the 8Fe cluster) with the simultaneous hydrolysis of a minimum of two MgATPs for each electron transferred (Mortenson et al., 1993). The oxidized FeP, with two MgADPs bound, is released from the MoFeP, and a second reduced FeP, with two associated MgATPs, binds to the one-electron-reduced MoFeP for a second round of MgATP hydrolysis and electron transfer (Hageman & Burris, 1978). Electrons, either one at a time or in pairs, are transferred to the FeMoco, where the bound substrate would be reduced by multiples of two electrons. This cycle would be repeated a sufficient number of times to fully reduce the substrate. The exact mode of substrate binding to FeMoco and the mechanism of electron transfer between the clusters of the MoFeP is not well understood (Orme-Johnson, 1992).

During the six-electron reduction of N_2 to form two NH_3, two protons are reduced by two electrons to form H_2. H_2 evolution by nitrogenase is an obligate part of the N_2 reduction mechanism, with a minimum of one H_2 evolved for each N_2 reduced even at high N_2 pressures (Simpson & Burris, 1984). In the absence of N_2 or other substrates, the total electron flow through nitrogenase goes to reduce protons to form H_2. In recent years, nitrogenase has been found to reduce a number of compounds in addition to N_2 and protons

†This work was supported by National Science Foundation Grant MCB-9315835 to L.C.S. and by a National Science Foundation Postdoctoral Fellowship to M.E.R.

* Corresponding author: Department of Chemistry and Biochemistry, Utah State University, Logan, UT 84322-0300. Phone: (801) 797-3964. Fax: (801) 797-3390. Internet: Seefeldt@cc.usu.edu.

‡ Utah State University.

§ Virginia Polytechnic Institute and State University.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1995.

[1] Abbreviations: FeP, iron protein of nitrogenase; MoFeP, molybdenum−iron protein of nitrogenase; CODH, carbon monoxide dehydrogenase; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

0006-2960/95/0434-5382$09.00/0

[see for reviews Burgess (1985) and Yates (1991)]. Analysis of affinities and rates of reduction of these alternative substrates has provided insights into the nature of the substrate binding site and the mechanism of reduction reactions catalyzed by nitrogenase. Examples of alternative substrates that are reduced by nitrogenase include N_2O, acetylene, hydrazine, azide, cyanide, allene, diazirine, nitriles, and isonitriles (Burris, 1991). A common property among these alternative substrates is the presence of N−N, N−O, N−C, or C−C double or triple bonds, which are reduced by multiples of two electrons by nitrogenase (Burgess, 1985). No examples have been reported to date of nitrogenase reduction of C−S or C−O double or triple bonds in alternative substrates.

In light of recent observations that carbonyl sulfide (COS) interacts with nitrogenase as an inhibitor of acetylene reduction (Madden et al., 1990) and that COS is a reducible substrate for the gas-utilizing enzyme carbon monoxide dehydrogenase (Ensign, 1995), it was of interest to examine the possibility that COS might be reduced by nitrogenase as an alternative substrate. In the present work, we demonstrate that nitrogenase can catalyze the two-electron reduction of a new class of compounds, namely, the C=S bond in COS to form the products CO and H_2S and the C=O bond of carbon dioxide (CO_2) to form the products CO and H_2O. These results represent the first examples of nitrogenase reduction of C=S and C=O bonds in alternative substrates. Finally, we also demonstrate that the COS and CO_2 analog carbon disulfide (CS_2) is a rapid-equilibrium inhibitor of nitrogenase substrate reduction reactions and total electron flow.

EXPERIMENTAL PROCEDURES

Materials

Hemoglobin was obtained from Pentex, Inc. (Kankakee, IL). COS (97.5%) was purchased from Matheson (Chicago, IL), while all other gases were purchased from Liquid Air Corp. (Walnut Creek, CA). Argon was purified of traces of oxygen by passage over a heated copper catalyst. Carbon disulfide (99.99%) was from EM Science (Gibbstown, NJ). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Methods

Nitrogenase Proteins. Nitrogenase MoFeP and FeP were purified from *Azotobacter vinelandii* cells as described previously and were homogeneous as determined by Coomassie staining of SDS−polyacrylamide gels (Seefeldt et al., 1992; Seefeldt & Mortenson, 1993). Specific activities for the nitrogenase component proteins were 2440 nmol of acetylene reduced min^{-1} (mg of MoFeP)$^{-1}$ and 2220 nmol of acetylene reduced min^{-1} (mg of FeP)$^{-1}$. Protein concentrations were determined by a modified Biuret method with bovine serum albumin as standard (Chromy et al., 1974).

Acetylene and Proton Reduction Assays. Acetylene and proton reduction rates were determined essentially as previously described with 10 mM dithionite ($Na_2S_2O_4$) as the reductant (Seefeldt et al., 1992). Acetylene reduction assay mixtures contained a gas phase of 8.4% (8.5 kPa) acetylene (unless noted otherwise) with the remainder as argon. Acetylene and ethylene were separated and ethylene was quantified by gas chromatography on a Shimadzu GC-8A with a flame ionization detector fitted with a 1 ft × 1/8 in. (30 cm × 0.3 cm) PoraPak N column with nitrogen as the carrier gas. The chromatograph was operated isothermally with an injector/detector temperature of 180 °C and a column temperature of 110 °C. Proton reduction assays were done under argon, and the product H_2 was quantified by gas chromatography on a Shimadzu GC-8 with a thermal conductivity detector fitted with a 2 ft × 1/8 in. (60 cm × 0.3 cm) molecular sieve 5A column with argon as the carrier gas (Seefeldt & Ensign, 1994). The chromatograph was operated isothermally with an injector/detector temperature of 100 °C, a column temperature of 50 °C, and a carrier gas flow rate of 15 mL/min.

Spectrophotometric Assay of COS and CO_2 Reduction. CO formation catalyzed by nitrogenase reduction of COS or CO_2 was quantified from changes in the absorption spectrum of hemoglobin as described (Bonam et al., 1984; Kumar et al., 1994). All spectrophotometric measurements were performed in 2.2-mL (1-cm path length) quartz cuvettes that had been modified to maintain a defined gas atmosphere. A quartz top was fused onto the cuvettes to accommodate a 13-mm butyl rubber septum and a crimp aluminum seal. The standard nitrogenase assay solution consisted of 83 mM TES, pH 7.0, or 83 mM Bis-Tris, pH 6.5, and 1 mg/mL bovine serum albumin, 0.15 mg/mL creatine phosphokinase, 2.7 mM MgATP, 4.5 mg/mL phosphocreatine, 0.2 mg/mL^{-1} hemoglobin and 8.3 mM dithionite ($Na_2S_2O_4$). A 1.2-mL aliquot of argon-purged assay solution was transferred to a cuvette that had been purged with argon. COS was added to the cuvette either as a gas or as assay buffer saturated with COS. CO_2 was added as a combination of CO_2 gas and 0.5 M $NaHCO_3$ under argon in a ratio of 7.5 vol of CO_2 to 1 vol of $NaHCO_3$ in order to maintain the pH at 6.5. For both COS and CO_2, the gases were allowed to equilibrate with the liquid phase for a minimum of 5 min with shaking. The concentration of each gas in solution was calculated from the known solubilities at the assay temperature. MoFeP and FeP were added to initiate the reactions. Spectrophotometric measurements were performed on a Hewlett-Packard 8452A diode-array spectrophotometer interfaced to a personal computer for data acquisition.

The pH optimum for COS reduction by nitrogenase was determined in the CO binding assay except that the buffer was changed to include a mixture of the buffers Bis-Tris, TES, and Tris, each at 33 mM. The pH of the buffer was adjusted with either NaOH or HCl.

CS_2 Inhibition of Nitrogenase Reduction Reactions. CS_2 inhibition of nitrogenase acetylene or proton reduction reactions was performed by addition of CS_2 to the assay vial prior to the addition of MoFeP and FeP. CS_2 was added from a 1 M stock solution diluted in dimethyl sulfoxide. CS_2 inhibition of COS reduction by nitrogenase was determined by the addition of CS_2 to the hemoglobin CO binding assay during the course of the assay. Control reactions in which DMSO was added alone showed no inhibition.

Numeric Constants and Data Analysis. The concentrations of COS (20.1 mM), acetylene (37.5 mM), and CO (1.0 mM) in aqueous solutions at 30 °C were determined from published Bunsen solubility coefficients (Wilhelm et al., 1977). CS_2 (16.6 M) was diluted to a 1 M stock solution in dimethyl sulfoxide immediately prior to use. The concentration of CO_2 in equilibrium with HCO_3^- at pH 6.5 was

calculated from the Henderson−Hasselbalch relationship with a pK_a of 6.3.

Kinetic constants (K_m and V_{max}) for COS and CO_2 were determined by nonlinear regression analysis using the program Igor (WaveMetrics, Inc., Lake Oswego, OR). Inhibition constants (K_{i1} and K_{i2}) for CS_2 were calculated from nonlinear regression fits to the equation for mixed or noncompetitive inhibition (Segel, 1975; Cleland, 1979; Cornish-Bowden, 1979).

RESULTS

Carbonyl Sulfide as a Reducible Substrate For Nitrogenase. Nitrogenase will reduce a number of relatively small compounds that contain double and triple bonds (Burgess, 1985). In all cases, the reduction reactions appear to occur as multiples of two electrons. Despite the range of compounds that have been shown to be reduced by nitrogenase, no examples have been reported to date of nitrogenase reduction of C−O or C−S bonds. A recent report found that COS could interact with nitrogenase as an inhibitor of acetylene reduction, suggesting that COS can bind to the nitrogenase active site (Madden et al., 1990). This observation, coupled with a recent observation that CODH from *Rhodospirillum rubrum* (Ensign, 1995) can reduce COS, suggested that nitrogenase might catalyze the reduction of COS according to the reaction in eq 2.

$$COS + 2H^+ + 2e^- \rightarrow CO + H_2S \qquad (2)$$

To test this possibility, we employed a hemoglobin CO binding assay which can be used to detect CO formation in real time using a spectrophotometer. This assay takes advantage of the fact that reduced hemoglobin binds CO rapidly and with high affinity, resulting in characteristic changes in the hemoglobin visible absorption spectrum, which can be used to quantify CO production (Bonam et al., 1984; Kumar et al., 1994). An additional advantage of the hemoglobin assay is that the CO produced is bound as carboxyhemoglobin and thus is not available to act as an inhibitor of nitrogenase. CO is known to be a potent inhibitor of all nitrogenase reduction reactions except for proton reduction (Hwang et al., 1973). Figure 1 illustrates absorption spectra of hemoglobin taken at different times after addition of nitrogenase FeP and MoFeP to a hemoglobin CO binding assay mixture containing MgATP, dithionite, and COS as a substrate. Dithionite served both to reduce hemoglobin to the ferrous state required for CO binding and as the source of electrons for nitrogenase reduction reactions. As can be seen in Figure 1, a time-dependent change in the hemoglobin absorption spectrum was observed under these conditions. These changes were identical to changes seen when CO was added directly to the hemoglobin assay in the absence of nitrogenase, confirming that the changes in the absorption spectrum resulted from CO formation. These results qualitatively revealed that nitrogenase could reduce COS to form CO. To quantify this reaction, the change in the hemoglobin absorption spectrum at 418 nm was routinely used to monitor, in real time, CO formation catalyzed by nitrogenase reduction of COS (Figure 2).

It was first necessary to determine whether the CO formation reaction was the result of nitrogenase catalysis. Figure 2 illustrates that the formation of CO from COS was

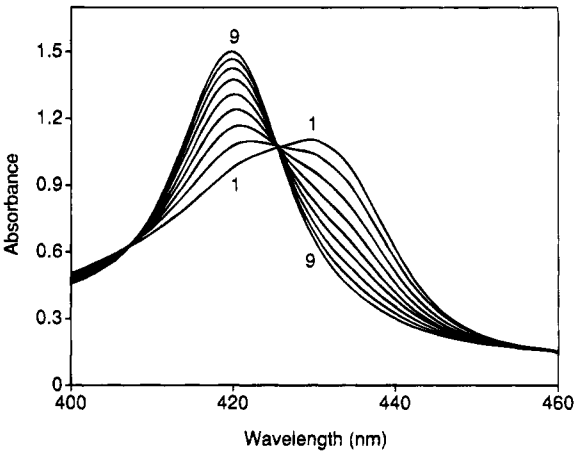


FIGURE 1: Time-dependent formation of carboxyhemoglobin catalyzed by nitrogenase reduction of COS. A hemoglobin CO binding assay was performed as described in Experimental Procedures. A 1.2-mL aliquot of COS-saturated assay solution containing 83 mM TES buffer, pH 7.0, 0.2 mg/mL hemoglobin, MgATP, a MgATP-regenerating system, and 8.3 mM dithionite was placed into a 2.2-mL quartz cuvette with 1 mL of Ar gas phase (101 kPa). The reaction was initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. Absorption spectra were recorded prior to the addition of nitrogenase (trace 1) and at 1-min intervals following the addition of nitrogenase (traces 2−9). The assay was done at 30 °C. The spectrum of the assay mixture recorded prior to the addition of nitrogenase or hemoglobin was subtracted from all spectra.

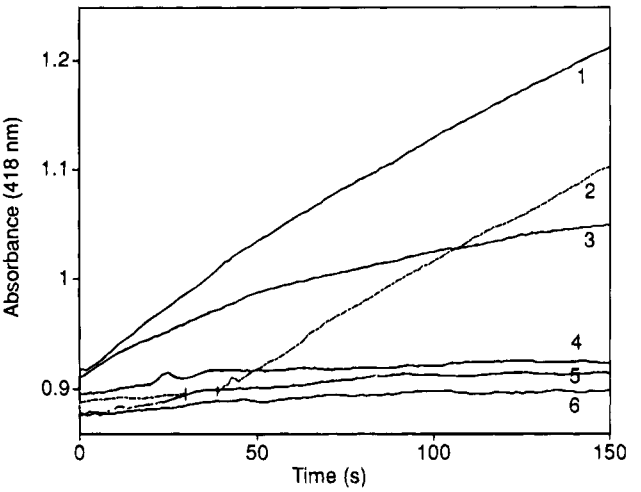


FIGURE 2: Spectrophotometric assay of CO formation catalyzed by nitrogenase reduction of COS. Hemoglobin CO binding assays were performed as outlined in Experimental Procedures. A 1.2-mL aliquot of assay solution (with 83 mM TES buffer, pH 7.0) was placed into a 2.2-mL cuvette under 101 kPa of Ar and 50 kPa of COS. Assays were initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. The assay conditions were as follows: trace 1, complete assay mixture; trace 2, assay initiated without MgATP followed by the addition of MgATP to 3 mM at 30 s; trace 3, assay mixture without the MgATP-regenerating enzyme creatine phosphokinase; trace 4, assay mixture without MoFeP; trace 5, assay mixture without MgATP; trace 6, assay mixture without FeP. All reactions were done at 30 °C. The change in absorbance at 418 nm as a function of time was recorded.

absolutely dependent upon the presence of both nitrogenase protein components (FeP and MoFeP), MgATP, and COS (Figure 2). This indicates that CO formation from COS was dependent on active nitrogenase and was not a result of a contaminating protein in one of the assay components. The rate of CO formation from COS reduction was also found to be proportional to the quantity of nitrogenase proteins added. As further evidence that the reaction was catalyzed

by nitrogenase, MgADP inhibition of CO formation was observed. MgADP is known to be a competitive inhibitor of MgATP binding and of substrate reduction reactions catalyzed by nitrogenase (Walker & Mortenson, 1974). Thus, in the absence of a MgATP regenerating system, MgADP is formed as a result of MgATP hydrolysis by nitrogenase, resulting in product inhibition of further MgATP hydrolysis and substrate reduction. To prevent this inhibition, the normal assay mixture includes phosphocreatine and phosphocreatine kinase, which phosphorylate the MgADP formed, regenerating MgATP. When the MgATP-regenerating system was omitted from the nitrogenase COS reduction assay, a time-dependent decrease in the rate of CO formation was observed. This is consistent with the time-dependent formation of MgADP acting as an inhibitor of nitrogenase reduction of COS. This result provided further evidence that the CO formation observed was indeed catalyzed by nitrogenase.

It was next necessary to rule out non-enzymatic reduction of COS catalyzed by H_2. It has been reported that H_2, at high temperatures, can reduce COS to CO and H_2S (Ferm, 1957). Since nitrogenase will reduce protons to produce H_2 in the absence of other substrates, it was possible that the H_2 produced by nitrogenase could be responsible for non-enzymatic reduction of COS. To test this possibility, a complete COS assay solution was prepared in the absence of nitrogenase, but in the presence of 101 kPa of H_2. The rate of CO formation was monitored spectrophotometrically and showed no CO formation above the background rate. This result confirmed that the CO formation was the result of the direct reduction of COS by nitrogenase and not the result of non-enzymatic reaction with H_2.

Kinetics of COS Reduction by Nitrogenase. We next examined the pH dependence of nitrogenase reduction of COS. The rate of COS reduction was determined at different pH values in a mixed buffer system over a range from pH 6.0 to 8.5. Under these conditions, a clear maximum rate of COS reduction was observed at pH 6.5 with lower rates observed at pH values both above and below 6.5. This same basic pH profile was observed for nitrogenase reduction of acetylene. All remaining kinetic studies were performed at pH 6.5.

The kinetics of nitrogenase reduction of COS were examined. Figure 3 shows the relationship between COS concentration and nitrogenase COS reduction rates. The results demonstrate that COS reduction by nitrogenase is saturable with respect to COS concentration, and fitting of the data in Figure 3 to the Michaelis–Menten equation provided a V_{max} of 37.2 ± 2.0 nmol of CO min^{-1} (mg of protein)$^{-1}$ and a K_m for COS of 3.1 ± 0.6 mM. A 2:1 molar ratio of FeP to MoFeP was used in these assays, as previous studies have demonstrated that this ratio results in the maximum rates of two-electron reduction of substrates (Rubinson et al., 1983).

In nitrogenase reduction of COS observed in the hemoglobin CO binding assay, dithionite served as the source of electrons for the reduction of COS. Dithionite is commonly used as the source of electrons for nitrogenase reduction reactions. Dithionite does not directly reduce the MoFeP for substrate reduction, but rather reduces the oxidized FeP after it releases from the MoFeP. Only the reduced FeP has been shown to transfer electrons to the MoFeP for substrate reduction. It was of interest to determine whether dithionite

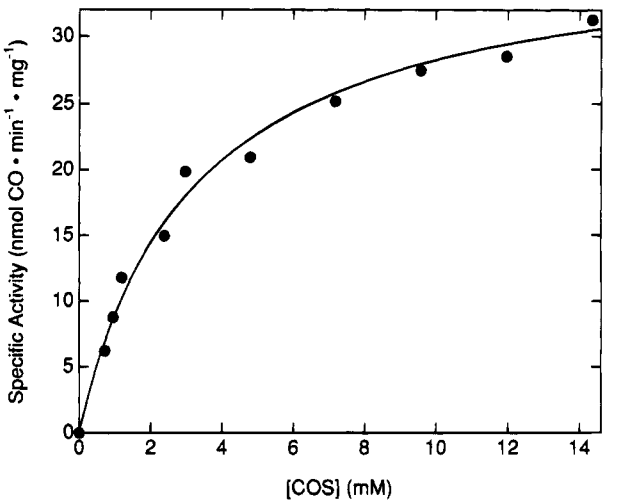


FIGURE 3: Effects of COS concentration on the rate of nitrogenase-catalyzed CO formation. The hemoglobin CO binding assay was as described in Experimental Procedures. A 2.1-mL aliquot of assay solution (with 83 mM Bis-Tris, pH 6.5) was added to a 2.2-mL quartz cuvette. Different COS concentrations were achieved by combining COS-saturated assay solution with Ar-saturated assay solution to give a final volume of 2.1 mL. The reaction was initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. All reactions were at 30 °C. The initial velocity is reported as nmol of CO produced min^{-1} (mg of FeP)$^{-1}$. The specific activity for CO formation is plotted against the concentration of COS in solution. The K_m (3.1 ± 0.6 mM) and V_{max} [37.2 ± 2.0 nmol of CO min^{-1} (mg of FeP)$^{-1}$] values were determined from nonlinear least squares fits to the equation for a hyperbola.

reduction of the FeP in the COS reduction assay was rate limiting. To test this possibility, reduced methyl viologen (100 μM) was included in the COS reduction assay as an alternative reductant for the reaction. Reduced methyl viologen will reduce FeP and will support nitrogenase substrate reduction reactions. The addition of methyl viologen to the COS reduction assay was found to have no effect on the rates of CO formation catalyzed by nitrogenase. This is consistent with previous results for nitrogenase substrate reduction reactions which have demonstrated that reduction of the FeP is not rate limiting (Hageman & Burris, 1978). The current model suggests that the release of the oxidized FeP–2MgADP complex from the MoFeP is the rate limiting step for proton and N_2 reduction (Mortenson et al., 1993).

COS Interaction with Nitrogenase. Analyses of the interactions of alternative substrates with nitrogenase have suggested that different substrates can interact either at different sites on the enzyme or with different oxidation states (Hwang et al., 1973; Burgess, 1985). The definition of these different binding sites is often derived from an examination of the competition between two or more substrates for binding to nitrogenase. For example, acetylene is known to block both N_2 reduction and proton reduction by nitrogenase (Schollhorn & Burris, 1967; Rivera-Ortiz & Burris, 1975). In contrast, N_2 will compete with acetylene reduction, but not with proton reduction. As one way to begin to define the nature of COS interaction with nitrogenase, it was of interest to examine the potential effects of other substrates on COS reduction rates. Figure 4 demonstrates the effects of acetylene, N_2, and H_2 on nitrogenase reduction of COS. As can be seen, both acetylene and N_2 inhibited COS reduction, with 67% inhibition observed for 0.63 mM acetylene or 0.6 mM N_2. Hydrogen is a specific inhibitor

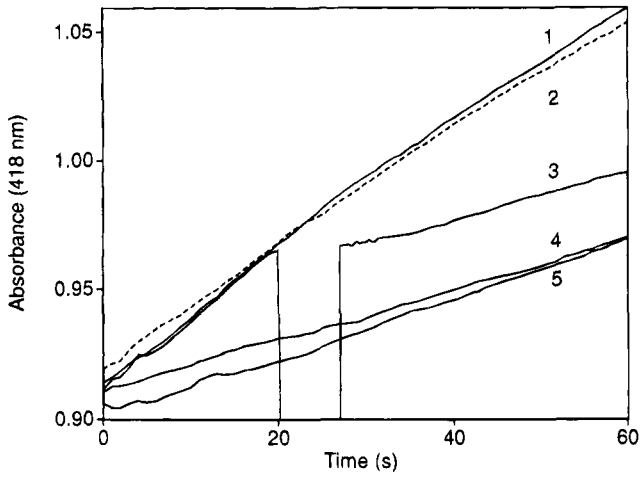


FIGURE 4: Effects of H_2, C_2H_2, and N_2 on nitrogenase COS reduction rates. Hemoglobin CO binding assays were as described in Experimental Procedures. A 1.2-mL aliquot of assay solution was added to a 2.2-mL quartz cuvette with either 101 kPa of Ar (trace 1), 101 kPa of H_2 (trace 2), 101 kPa of Ar with 20 μL of C_2H_2-saturated solution (750 nmol in solution) added 20 s after initiation of the assay (trace 3), 101 kPa of Ar with 750 nmol of C_2H_2 present at the beginning of the assay (trace 4), or 101 kPa of N_2 (trace 5). In all cases, COS was added as an overpressure of 50 kPa. The assays were initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. All assays were done at 30 °C. The increase in absorbance at 418 nm as a function of time was recorded.

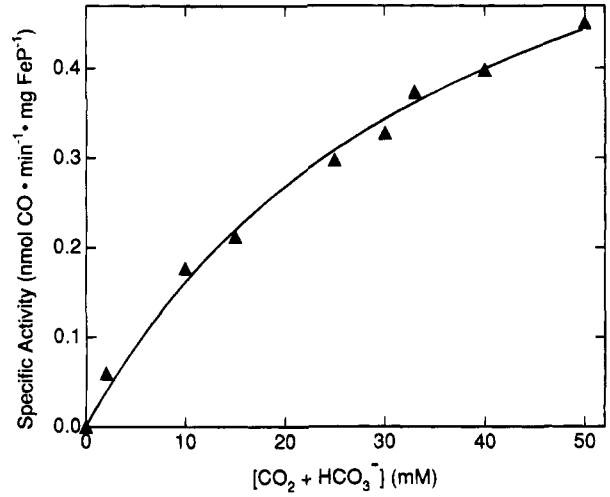


FIGURE 5: Effects of CO_2 concentration on the rate of nitrogenase-catalyzed CO formation. Hemoglobin CO binding assays were performed as outlined in Experimental Procedures. A 1.2-mL aliquot of assay solution (with 83 mM Bis-Tris buffer, pH 6.5) was placed into a 2.2-mL cuvette under 101 kPa of Ar. CO_2 was added as a combination of CO_2 gas and 0.5 M $NaHCO_3$ under argon in a ratio of 7.5 vol of CO_2 to 1 vol of $NaHCO_3$ in order to maintain the pH at 6.5. Assays were initiated by the addition of 700 μg of MoFeP and 345 μg of FeP and were performed at 30 °C. The initial velocity is reported as nmol of CO produced min^{-1} (mg of FeP)$^{-1}$. The specific activity for CO formation is plotted against the concentration of CO_2 and bicarbonate added. The K_m for CO_2 plus HCO_3^- (38.4 ± 6.4 mM) and the V_{max} [0.8 ± 0.07 nmol of CO min^{-1} (mg of FeP^{-1})] were determined from nonlinear least squares fit to the equation for a hyperbola.

of N_2 reduction by nitrogenase but not of reduction of other alternative substrates such as acetylene (Burgess, 1985). Figure 4 reveals that H_2 concentrations up to 0.6 mM had no inhibitory effect on COS reduction by nitrogenase.

Previous results have demonstrated that COS does inhibit acetylene reduction by nitrogenase, but not proton reduction (Madden et al., 1990). These results are now explainable by the fact that COS is a substrate for nitrogenase reduction and that acetylene, N_2, and COS all seem to compete for binding to the same site for reduction. We also confirmed the earlier observation that COS did not inhibit proton reduction by nitrogenase up to a concentration of 5 mM COS in solution.

Carbon Dioxide as a Reducible Substrate for Nitrogenase. The discovery that nitrogenase could reduce COS suggested the possibility that the analogous compound, CO_2, might also be a substrate. This possibility was further suggested by the fact that the metalloenzyme CODH can reduce both CO_2 and COS. Thus, it was of interest to determine whether nitrogenase could catalyze the two-electron reduction of CO_2 according to the reaction in eq 3, which would be analogous to the reduction of COS described in eq 2.

$$CO_2 + 2H^+ + 2e^- \rightarrow CO + H_2O \qquad (3)$$

To test this possibility, the hemoglobin CO binding assay was again used to quantify CO produced from the reduction of CO_2. A time-dependent production of CO was observed that was dependent on the presence of both nitrogenase component proteins, MgATP, and CO_2, confirming that nitrogenase catalyzes the reduction of CO_2 to CO.

The rate of CO production catalyzed by nitrogenase was dependent on the concentration of total carbonate in solution as shown in Figure 5. The results demonstrate that CO_2 reduction by nitrogenase is saturable with respect to CO_2 concentration, and fitting the data in Figure 5 to the Michaelis–Menten equation provided a V_{max} of 0.8 ± 0.07 nmol of CO min^{-1} (mg of protein)$^{-1}$ and a K_m for total carbonate species [CO_2 + HCO_3^-] of 38.1 ± 6 mM. Since CO_2 is in equilibrium with HCO_3^- in solution, the exact species that is reduced by nitrogenase is not known, although CO_2 seems like the most reasonable substrate. Assuming CO_2 as the substrate for nitrogenase, a K_m for CO_2 of 23.3 ± 3.7 mM was determined from the data in Figure 5.

Carbon Disulfide as an Inhibitor of Nitrogenase Reduction Reactions. Given the interaction of CO_2 and COS with nitrogenase as substrates, it was of interest to determine whether the structural analog CS_2 might also interact with nitrogenase. CS_2 is known to be a ligand to metals, and it has been shown to interact with several metalloenzymes, including CO- and CO_2-utilizing enzymes (Hyman et al., 1990; Anderson & Lindahl, 1994; Kumar et al., 1994). Therefore, CS_2 was examined as a potential inhibitor of nitrogenase reduction reactions. The effects of increasing CS_2 concentration on the nitrogenase reduction of acetylene are illustrated in Figure 6. From a Dixon plot of the data, it was clear that CS_2 was a rapid-equilibrium inhibitor of acetylene reduction catalyzed by nitrogenase. As the Dixon plot does not distinguish between competitive and mixed-type inhibition, the data in Figure 6 were plotted as the slope of each inhibition line against the reciprocal of substrate (C_2H_2) concentration (Figure 6, inset) (Cornish-Bowden, 1979). The resulting line intersected the y-axis above zero, consistent with mixed-type inhibition with respect to C_2H_2. Fitting of the data to the equation for mixed-type inhibition (Cleland, 1979) revealed values of K_{i1} of 2.9 ± 0.6 mM and K_{i2} of 12.1 ± 2.0 mM.

CO is an inhibitor of acetylene reduction, but it is not an inhibitor of proton reduction catalyzed by the wild-type Mo-

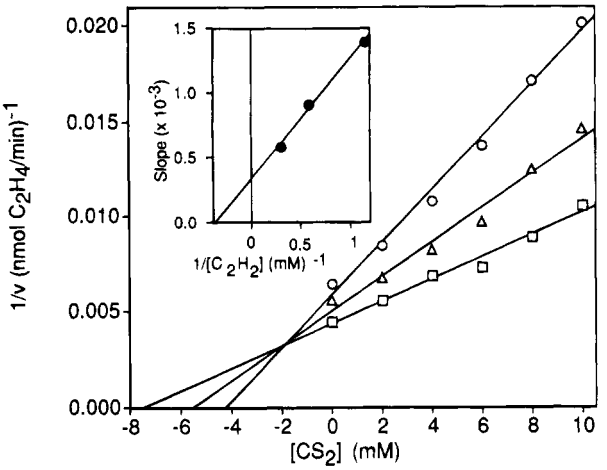


FIGURE 6: CS_2 inhibition of nitrogenase-catalyzed acetylene reduction. Acetylene reduction assays were performed as described in Experimental Procedures except that acetylene was added to a final concentration in solution of 3.48 (□), 1.74 (△), or 0.87 mM (○). CS_2 was added to the indicated concentration from a 1 M stock made in DMSO. The reaction was initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. The reciprocal of the rate of acetylene reduction was plotted against the concentration of CS_2 at each acetylene concentration (Dixon plot). A plot of the slope of each line against the reciprocal of the acetylene concentration (inset) indicated mixed-type inhibition. A fit of the data to the equation for mixed inhibition resulted in $K_{i1} = 2.9 \pm 0.6$ mM and $K_{i2} = 12.1 \pm 2$ mM.

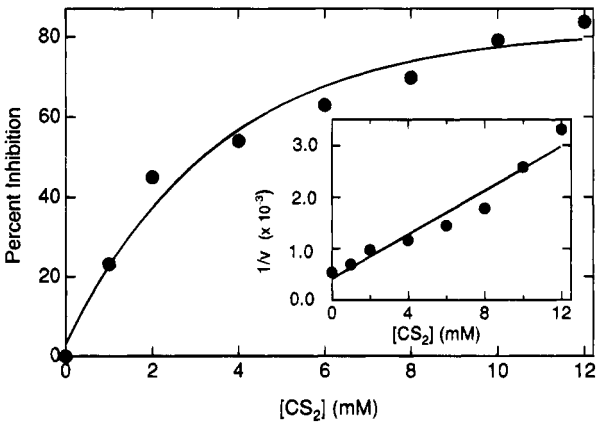


FIGURE 7: CS_2 inhibition of nitrogenase-catalyzed proton reduction. Proton reduction assays were performed as described in Experimental Procedures. CS_2 was added to the indicated final concentration from a 1 M stock made in DMSO. The reaction was initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. The percent inhibition of the noninhibited proton reduction rate was plotted against the concentration of CS_2. Inset: The reciprocal of the inhibited rates plotted against the concentration of CS_2 (Dixon plot).

based nitrogenase. In contrast, many other compounds (including alternative substrates) have been shown to inhibit both acetylene and proton reduction reactions catalyzed by nitrogenase (Burgess, 1985). Since COS and CO_2 did not inhibit proton reduction and CO does not inhibit proton reduction, it was of interest to see whether CS_2 could inhibit proton reduction. To test this possibility, increasing concentrations of CS_2 were included in a proton reduction assay and the rate of hydrogen formation was determined. The results from such an experiment are presented in Figure 7 and illustrate that CS_2 inhibits proton reduction by nitrogenase. A Dixon plot of the data presented in Figure 7 (inset) revealed that CS_2 was a rapid-equilibrium inhibitor of proton reduction catalyzed by nitrogenase. The type of CS_2

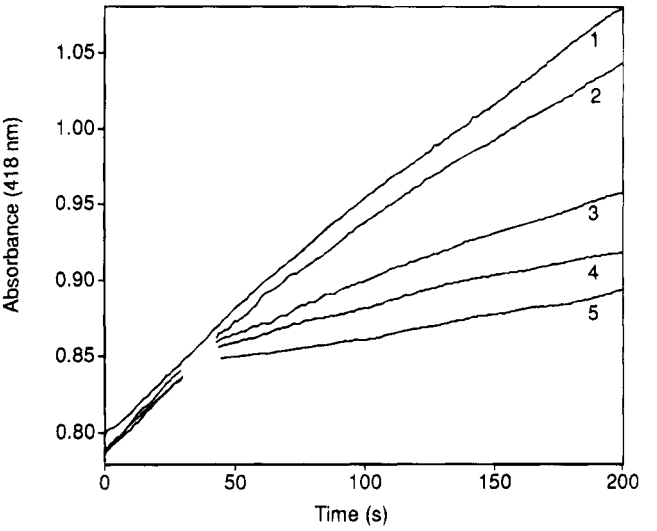


FIGURE 8: CS_2 inhibition of nitrogenase-catalyzed COS reduction. Hemoglobin CO binding assays were performed as outlined in Experimental Procedures. A 1.2-mL aliquot of assay solution (with 83 mM TES buffer, pH 7.0) was placed into a 2.2-mL cuvette under 101 kPa of Ar and 50 kPa of COS. Assays were initiated by the addition of 256 μg of MoFeP and 117 μg of FeP. CS_2 was added at 40 s into the assay to the following final concentrations: trace 1, 0 mM; trace 2, 1 mM; trace 3, 2.5 mM; trace 4, 5 mM; trace 5, 8 mM. All reactions were done at 30 °C. The change in absorbance at 418 nm as a function of time was recorded.

inhibition with respect to proton reduction was not determined since changing the substrate concentration (protons in this case) complicates the determination of the inhibited rates as a result of pH effects on the protein. The concentration of CS_2, however, that resulted in 50% inhibition of proton reduction at pH 6.5 was estimated to be 3 mM, which is the same as K_{i1} found for CS_2 inhibition of acetylene reduction. Thus, in contrast to COS, CO_2, and CO, CS_2 was found to inhibit proton reduction by nitrogenase.

Finally, CS_2 was tested as a potential inhibitor of nitrogenase reduction of COS. Figure 8 illustrates the effects of increasing CS_2 concentrations on the COS reduction catalyzed by nitrogenase. Like CS_2 inhibition of acetylene or proton reduction, 3 mM CS_2 resulted in approximately 50% inhibition of the COS reduction rate. The rapid inhibition of the carboxyhemoglobin formation rates shown in Figure 8 following the addition of CS_2 suggest that CS_2 is a rapid-equilibrium inhibitor of COS reduction by nitrogenase.

DISCUSSION

In the present work, we have extended the range of substrates that can be reduced by nitrogenase to include COS and CO_2. These substrates represent the first examples of nitrogenase reduction of C=O and C=S bonds. CS_2 was also shown to be a rapid-equilibrium inhibitor of acetylene, proton, and COS reduction by nitrogenase.

COS and CO_2. The observation that nitrogenase can reduce COS and CO_2 by two electrons fits with earlier observations on the two-electron reduction of other substrates by nitrogenase (Burgess, 1985; Yates, 1991). Two such reactions, the reduction of N_2O and azide, appear to be quite similar to the reduction of COS and CO_2 (Scheme 1).

In the case of nitrogenase reduction of N_2O, the N=O double bond is reduced by two electrons and cleaved, resulting in the products N_2 and H_2O (Jensen & Burris, 1986; Liang & Burris, 1988). The two-electron reduction of azide

Scheme 1

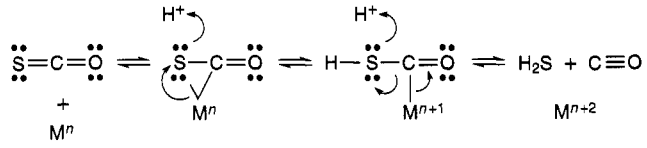
$$N{=}N{=}N^- + 2e^- + 3H^+ \rightleftharpoons N{\equiv}N + H_3N$$

$$N{=}N{=}O + 2e^- + 2H^+ \rightleftharpoons N{\equiv}N + H_2O$$

$$S{=}C{=}O + 2e^- + 2H^+ \rightleftharpoons C{\equiv}O + H_2S$$

$$O{=}C{=}O + 2e^- + 2H^+ \rightleftharpoons C{\equiv}O + H_2O$$

Scheme 2



is similar, with the two-electron reduction of the N=N double bond resulting in cleavage of the bond and formation of the products N_2 and NH_3 (Rubinson et al., 1985). The reduction reactions proposed in the present work for COS and CO_2 are analogous to the above reactions. In the case of COS, nitrogenase was found to catalyze the two-electron reduction and cleavage of the C=S bond, resulting in the products CO and H_2S. Likewise, the reduction and cleavage of the C=O bond of CO_2 resulted in the formation of CO and H_2O. The possibility that nitrogenase could catalyze reactions with COS and CO_2 different from those proposed in Scheme 1 cannot be ruled out. However, the observation of CO formation and analogy to other two-electron reduction reactions make the proposed reaction mechanism seem most likely. Likewise, the products of the reduction of COS and CO_2 (CO, H_2O, and H_2S) would not be expected to be substrates for further reduction by nitrogenase. The lack of reduction of CO by nitrogenase is well documented and not completely explainable (Burgess, 1985).

It is noteworthy that COS was a much better substrate for nitrogenase reduction than was CO_2 in terms of both kinetic affinity (K_m) and maximum turnover rate (k_{cat}). The K_m for COS (3.1 mM) was 7.5 times lower than that for CO_2 (23.3 mM). These values can be compared to the K_m values for other substrates for nitrogenase. The K_m for COS of 3.1 mM is very near the K_m for several alternative substrates such as N_2O (K_m = 5 mM) (Jensen & Burris, 1986), HCN (K_m = 4.5 mM), (Li et al., 1982), acetylene (K_m = 0.4 mM) (Davis et al., 1975), and azide (K_m = 0.9 mM) (Rubinson et al., 1985). In contrast, the K_m for the physiologically relevant substrate N_2 (K_m = 0.08 mM) is significantly lower. The K_m for CO_2 of 23.3 mM is much higher than for most other alternative substrates. A maximum turnover rate (k_{cat}) for COS reduction of 0.16 s^{-1} can be compared to the maximum turnover rate for N_2, proton, and acetylene reduction of 8.3 s^{-1}. The turnover rate for CO_2 reduction was much slower (0.0033 s^{-1}) than other substrate reduction reactions catalyzed by nitrogenase. The catalytic efficiencies (k_{cat}/K_m) for COS (3.1 mM^{-1} s^{-1}) and CO_2 (0.0085 mM^{-1} s^{-1}) reduction are significantly lower than that for N_2 (104 mM^{-1} s^{-1}). The catalytic efficiency for COS reduction was closer to that for acetylene (21 mM^{-1} s^{-1}).

Given the obvious similarities in the reduction reactions catalyzed by nitrogenase outlined in Scheme 1, it seems likely that a common mechanism for these two-electron reduction reactions might exist. Scheme 2 outlines one likely mechanism for how COS and CO_2 might be reduced by nitrogenase. In this scheme, M would represent the FeMoco of MoFeP. Several lines of evidence suggest that substrates are bound to and reduced at the FeMo cofactor of the MoFeP. Recent X-ray crystal structures for the MoFePs from *A. vinelandii* (Kim & Rees, 1992a,b) and *Clostridium pasteurianum* (Bolin et al., 1993; Kim et al., 1993) have provided a model for the FeMo cofactor of nitrogenase, although the site and mode of binding of substrates remain unknown. One attractive model for how substrates might bind to FeMoco suggests that substrates bind in the plane of four iron atoms of FeMoco (Dance, 1994). Thus, in Scheme 2, M would represent these four Fe atoms. The C=O or C=S portion of CO_2 or COS would bind between the four Fe atoms and would be reduced by two electrons. The same basic scheme for COS and CO_2 activation outlined in Scheme 2 could be drawn for N_2O and azide reduction.

One other class of enzymes has been shown to reduce both CO_2 and COS according to the reaction shown in Scheme 1, namely, carbon monoxide dehydrogenases (CODHs) from *R. rubrum* (Ensign, 1995) and *Clostridium thermoaceticum* (Gorst, 1991; Kumar et al., 1994). Therefore, it is of interest to compare and contrast the reduction of COS and CO_2 by nitrogenase and CODH. Carbon monoxide dehydrogenases (CODH) are Ni–Fe metalloenzymes which occur in a variety of bacteria where they catalyze the oxidation of CO to form CO_2. Recent reports have demonstrated that the CODH isolated from *C. thermoaceticum* can catalyze the reverse reaction, namely, the reduction of CO_2 to form CO and H_2O (Lindahl et al., 1990; Gorst, 1991; Kumar et al., 1994). In an accompanying paper (Ensign, 1995), it is demonstrated that the *R. rubrum* CODH can reduce CO_2 to CO and H_2O and COS to CO and H_2S. As demonstrated in the present work, nitrogenase is capable of catalyzing the two-electron reduction of COS and CO_2 via reactions analogous to those catalyzed by *R. rubrum* CODH. The *C. thermoaceticum* CODH has also been shown to reduce N_2O, like nitrogenase, by two electrons to N_2 and H_2O (Lu & Ragsdale, 1991). The similarity between the reactions catalyzed by these two classes of enymes can be contrasted with several other observations. While both nitrogenase and CODH are metalloenzymes, the active site metal clusters in the two enzymes are very different with a nickel–iron–sulfur site in CODH and the FeMoco in nitrogenase. While it seems likely that the general mechanism proposed in Scheme 2 is used by both classes of enzymes, M in each case is different. The kinetic affinities and turnover numbers for reduction of COS and CO_2 by the two classes of enzymes are also quite different. In general, the *R. rubrum* CODH has much lower K_m values for COS and CO_2 and much higher turnover numbers when compared to nitrogenase. The catalytic efficiency for the reduction of COS was 4600 times greater for CODH when compared to nitrogenase (240 mM^{-1} s^{-1} vs 0.052 mM^{-1} s^{-1}) and 1.7×10^6 greater for CO_2 reduction (240 mM^{-1} s^{-1} vs 0.000 14 mM^{-1} s^{-1}). It is interesting that the rates of reduction of COS for the two classes of enzymes were quite similar, with turnover numbers of 0.51 s^{-1} for CODH and 0.16 s^{-1} for nitrogenase. The differences between the enzymes are much greater for CO_2 reduction, with a turnover number of 45 s^{-1} for CODH compared to 0.0033 s^{-1} for nitrogenase. It is interesting that nitrogenase reduces COS faster than CO_2, while CODH reduces CO_2 faster than COS. This difference seems obvious from the fact that CO_2 is the product of the physiological reaction catalyzed by CODH, where it is not for nitrogenase. The

absolute requirement for MgATP hydrolysis for nitrogenase reduction of all substrates is clearly another significant difference between nitrogenase and CODH.

CS_2. A significant observation of the present work is that the COS and CO_2 analog CS_2 is an inhibitor of nitrogenase reduction reactions. CS_2 was found to be a rapid-equilibrium inhibitor of acetylene, proton, and COS reductions catalyzed by nitrogenase. With respect to acetylene reduction, CS_2 was found to be a mixed-type inhibitor. This observation is analogous to CO inhibition, where CO is noncompetitive with respect to acetylene reduction. Noncompetitive inhibition is a special case of mixed inhibition (Cornish-Bowden, 1979). The classic interpretation for mixed-type inhibition would suggest that CS_2 binds both to the free enzyme state (E) and to the enzyme−acetylene bound state (E−C_2H_2) with different affinities (K_{i1} and K_{i2}, where K_{i1} is for the formation of the E−CS_2 complex and K_{i2} is for formation of the E−C_2H_2−CS_2 complex) (Segel, 1975). The noncompetitive inhibition seen for CO would suggest that CO also binds to both E and E−C_2H_2, but that the affinities for each state are identical ($K_{i1} = K_{i2}$). In contrast to CO, however, CS_2 was found to inhibit proton reduction. In this regard, CS_2 appears to act more like some alternative substrates for nitrogenase. The possibility that CS_2 might be a substrate for nitrogenase reduction by a mechanism analogous to that presented in Scheme 2 has been considered, although this possibility has not been thoroughly examined. It is interesting that CS_2 inhibition of nitrogenase is kinetically different from COS inhibition, considering the similarities between the two compounds. This suggests that CS_2 is interacting with nitrogenase in a different way than COS or CO.

CS_2 interaction with the active site of nitrogenase is consistent with the observation that CS_2 forms complexes with many different metals (Ibers, 1982). In addition, CS_2 has been shown to inhibit several metalloenzymes, including the *C. thermoaceticum* CODH (Anderson & Lindahl, 1994; Kumar et al., 1994) and the *R. rubrum* CODH (Ensign, 1995). In the case of *C. thermoaceticum* CODH, CS_2 was found to alter the EPR spectrum of a nickel−iron active site (Kumar et al., 1994). CO binding to nitrogenase has been shown to result in changes in EPR signals in MoFeP (Davis et al., 1979). It will now be interesting to probe the effects of CS_2 on the EPR spectrum of MoFeP.

ACKNOWLEDGMENT

The authors thank Drs. Richard Holz and John Hubbard for helpful discussions.

REFERENCES

Anderson, M. E., & Lindahl, P. A. (1994) *Biochemistry 33,* 8702−8711.
Bolin, J. T., Ronco, A., Morgan, T. V., Mortenson, L. E., & Xuong, N. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 1078−1082.
Bonam, D., Murrel, S. A., & Ludden, P. W. (1984) *J. Bacteriol. 159,* 693−699.
Burgess, B. K. (1985) in *Metal Ions in Biology: Molybdenum Enzymes* (Spiro, T. G., Ed.) pp 161−220, John Wiley and Sons, New York.
Burris, R. H. (1991) *J. Biol. Chem. 266,* 9339−9342.
Chromy, V., Fischer, J., & Kulhanek, V. (1974) *Clin. Chem. 20,* 1362−1363.
Cleland, W. W. (1979) *Methods Enzymol. 63,* 103−138.
Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics,* Butterworths, London.
Dance, I. G. (1994) *Aust. J. Chem. 47,* 979−990.
Davis, L. C., Shah, V. K., & Brill, W. J. (1975) *Biochim. Biophys. Acta 403,* 67−78.
Davis, L. C., Henzl, M. T., Burris, R. H., & Orme-Johnson, W. H. (1979) *Biochemistry 18,* 4860−4869.
Dean, D. R., Bolin, J. T., & Zheng, L. (1993) *J. Bacteriol. 175,* 6737−6744.
Ensign, S. A. (1995) *Biochemistry 34,* 5372−5381.
Ferm, R. J. (1957) *Chem. Rev. 57,* 621−640.
Georgiadis, M. M., Komiya, H., Chakrabarti, P., Woo, D., Kornuc, J. J., & Rees, D. C. (1992) *Science 257,* 1653−1659.
Gorst, C. M. (1991) Ph.D. Thesis, University of Wisconsin−Milwaukee.
Hageman, R. V., & Burris, R. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75,* 2699−2702.
Hwang, J. C., Chen, C. H., & Burris, R. H. (1973) *Biochim. Biophys. Acta 292,* 256−270.
Hyman, M. R., Kim, C. Y., & Arp, D. J. (1990) *J. Bacteriol. 172,* 4775−4782.
Ibers, J. A. (1982) *Chem. Rev. 11,* 57−73.
Jensen, B. B., & Burris, R. H. (1986) *Biochemistry 25,* 1083−1088.
Kim, J., & Rees, D. C. (1992a) *Nature 360,* 553−560.
Kim, J., & Rees, D. C. (1992b) *Science 257,* 1677−1682.
Kim, J., & Rees, D. C. (1994) *Biochemistry 33,* 389−397.
Kim, J., Woo, D., & Rees, D. C. (1993) *Biochemistry 32,* 7104−7115.
Kumar, M., Lu, W. P., & Ragsdale, S. W. (1994) *Biochemistry 33,* 9769−9777.
Li, J., Burgess, B. K., & Corbin, J. L. (1982) *Biochemistry 21,* 4393−4402.
Liang, J., & Burris, R. H. (1988) *Biochemistry 27,* 6726−6732.
Lindahl, P. A., Munck, E., & Ragsdale, S. W. (1990) *J. Biol. Chem. 265,* 3873−3879.
Lowe, D. J., Fisher, K., & Thorneley, R. N. F. (1993) *Biochem. J. 292,* 93−98.
Lu, W. P., & Ragsdale, S. W. (1991) *J. Biol. Chem. 266,* 3554−3564.
Madden, M. S., Kindon, N. D., Ludden, P. W., & Shah, V. K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 6517−6521.
Mortenson, L. E., Seefeldt, L. C., Morgan, T. V., & Bolin, J. (1993) *Adv. Enzymol. 67,* 299−373.
Orme-Johnson, W. H. (1992) *Science 257,* 1639−1640.
Rees, D. C., Chan, M. K., & Kim, J. (1993) *Adv. Inorg. Chem. 40,* 89−119.
Rivera-Ortiz, J. M., & Burris, R. H. (1975) *J. Bacteriol. 123,* 537−545.
Rubinson, J. F., Corbin, J. L., & Burgess, B. K. (1983) *Biochemistry 22,* 6260−6268.
Rubinson, J. F., Burgess, B. K., Corbin, J. L., & Dilworth, M. J. (1985) *Biochemistry 24,* 273−283.
Schollhorn, R., & Burris, R. H. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58,* 213−216.
Seefeldt, L. C., & Mortenson, L. E. (1993) *Protein Sci. 2,* 93−102.
Seefeldt, L. C., & Ensign, S. A. (1994) *Anal. Biochem. 221,* 379−386.
Seefeldt, L. C., Morgan, T. V., Dean, D. R., & Mortenson, L. E. (1992) *J. Biol. Chem. 267,* 6680−6688.
Segel, I. H. (1975) *Enzyme Kinetics,* John Wiley and Sons, New York.
Simpson, F. B., & Burris, R. H. (1984) *Science 224,* 1095−1097.
Smith, B. E., & Eady, R. R. (1992) *Eur. J. Biochem. 205,* 1−15.
Walker, G. A., & Mortenson, L. E. (1974) *Biochemistry 13,* 2382−2388.
Wilhelm, E., Battino, R., & Wilcock, R. J. (1977) *Chem. Rev. 77,* 219−262.
Yates, M. G. (1991) in *Biological Nitrogen Fixation* (Stacey, G., Burris, R. H., & Evans, H. J., Eds.) pp 685−735, Chapman and Hall, New York.

BI942719N